

SQUIRRELL et al.  
Serial No. 09/529,722

mutations at amino acids 87 and 107 in the sequence of *E. coli* adenylate kinase.--

REMARKS

Reconsideration is requested.

Claims 19-32 have been canceled, without prejudice. Claims 33-46 have been added and are pending. Claims 33-46 are generally similar to now canceled claims 19-21 and 23-32. No new matter has been added.

The specification has been amended to correct inadvertent typographical errors.

No new matter has been added.

The title has been amended, as suggested by the Examiner on page 2 of the Office Action dated January 24, 2002 (Paper No. 17).

The Examiner's requirement for a newly executed Declaration is noted and the same will be filed under separate cover once received by the undersigned.

An indication of the acceptance of the drawings in the Examiner's next Action by either the Examiner or the Chief Draftsperson would be appreciated.

The objection to claim 27 noted in paragraph 4 on page 3 of Paper No. 17 is moot in view of the above. The objected-to language has not been repeated in the amended claims.

The Section 112, second paragraph, rejection of claims 19-32 stated on pages 3-4 of Paper No. 17 are moot in view of the above. The pending claims have been drafted with the Examiner's comments in mind and the pending claims are submitted to be definite.

Specifically, claim 25 has been rewritten as new claim 38 to include the recitation that the enzymatic activity of the luciferase is not adversely affected rather than incorporating the Examiner's suggestion. The claims have also been amended to include the recitation that the luciferase is a thermostable luciferase and the recitation of now canceled claim 22 (i.e., that the temperature conditions are elevated to temperatures of 37°C or more) have been included in new claim 33.

The claims are submitted to be definite.

The Section 112, first paragraph, rejection of claims 19-23 and 25-32, stated in paragraphs 13 and 14 of Paper No. 17 are moot in view of the above. The pending claims are submitted to be supported by an enabling disclosure which provides sufficient written description of the presently claimed invention, and consideration of the following in this regard is requested.

The applicants respectfully submit that the recitation of any particular luciferase or adenylate kinase should not be required as it is clear from the specification that the disclosed method is not dependent on or requires any specific thermostable luciferase and/or adenylate kinase containing a specific mutation at amino acids 87 or 107 in the sequence of *E. coli* adenylate kinase, as the method of the present invention is useful with any adenylate kinase enzyme.

With regard to the temperature conditions, the recitation of 37°C or more, makes it clear to an ordinarily skilled person that the temperature has to be at least 37°C in order to denature the adenylate kinase enzyme. It would be a matter of routine experimentation to identify the upper temperature limit at which the particular luciferase

enzyme becomes unstable. Similarly with regard to the pH conditions, an ordinarily skilled person knowing that by changing the pH conditions using the cells of the present invention would believe it to be a matter of routine experimentation to determine the pH conditions where the adenylate kinase would become unstable.

The Examiner is apparently under the impression that the claims, such as now-canceled claims 19 and 25, cover any luciferase which is stable under **any** pH or temperature conditions and any adenylate kinase which is unstable under **any** pH or temperature conditions. This is not, in fact, the case. The claims clearly cover a method for producing a luciferase, which is substantially free of adenylate kinase, wherein the host cell culture or the recovered luciferase is subjected to conditions of pH or temperature under which the adenylate kinase is denatured wherein the luciferase retains its activity. The claims cover conditions wherein adenylate kinase is denatured without denaturing the luciferase. The recitation of 37<sup>0</sup>C or more in claim 33, for example, clearly indicates as much.

It is believed that the scope of the claims is commensurate with the contribution to the art. In this regard, the applicants are the first to appreciate that instabilities that occur in mutant forms of proteins can be used in recombinant DNA technology to allow easier and better purification. This is especially advantageous in that it allows the removal of a particular contaminant protein from a thermostable enzyme. Therefore, as discussed above, the recitation of any particular luciferase or adenylate kinase would not reflect the applicants' contribution to the art which, given the broad applicability of the invention, should be rewarded with claim protection of at least the scope recited

above. Although other methods of removing contamination may have been known, none of these prior methods teach or suggest a method wherein particular enzymes may be targeted for removal.

The claims are submitted to be supported by an enabling disclosure which adequately describes the claimed invention.

The Section 103 rejection of claims 19-32 over EP 373962 in view of Belinga (J. Chomat A 695:33-40), Gilles (PNAS 83:5798-5802) and Kayijama (Biochemistry 32:13795-13799) is moot in view of the above. The pending claims are submitted to be patentable over the cited art and consideration of the following in this regard is requested.

The applicants believe that the Examiner has impermissibly used hindsight in an attempt to piece together the invention as claimed. This is particularly surprisingly since the Examiner stated in the Office Action dated 27 February 2001 (Paper No. 4) that that there is no specific teaching or suggestion in the prior art of a method of isolating a thermostable enzyme free of a contaminant by genetically modifying the contaminant for the purposes of creating a thermolabile mutant and using heat denaturation to remove contaminant activity from the thermostable enzyme. See, paragraph 10 of Paper No. 4.

The only newly cited art, i.e., Gilles, fails to cure the deficiencies of the previously cited art, which were previously appreciated by the Examiner.

The presently claimed invention is patentable over the cited prior art. In this regard, the problem to be solved by the present invention is the provision of a method for producing a thermostable luciferase enzyme free of adenylate kinase using genetic

modification. This problem is solved by the creation of a thermosensitive mutant adenylate kinase which is considered to be a contaminant of the thermostable enzyme and using heat denaturation or suitable pH conditions to remove the adenylate kinase from the thermostable luciferase.

Taking EP 0 373 962A first [Backman] as the closest cited prior art, the problem to be solved is a method of purifying a thermostable ligase or polymerase from unwanted contaminants. This is a different problem from that of the present invention, which relates to the removal of adenylate kinase activity from a culture containing a luciferase. It is not believed that an ordinarily skilled person would therefore have considered this document.

Even if an ordinarily skilled person were to have considered this document, it is not believed that he would have produced the present invention as it relates to a process in which highly thermostable enzymes, such as those obtainable from species such as *Thermus aquaticus*, which are intended for use in amplification reactions such as PCR, can be purified. These enzymes are expressed in a mesophilic cell such as *E. coli* and then purified by heating to extreme temperatures (80°C for 30 minutes is given) in order to denature contaminants.

The contaminants in this case are all other proteins native in the host cell. The method does not involve the use of **only** mutant forms of the contaminant proteins wherein particular proteins are targeted for removal. In particular, EP 0 373 962 A does not teach or suggest the use of recombinant DNA technology to introduce the mutant adenylate kinase gene into the host cell. The presently claimed invention is therefore

inventive and patentable over EP 0 373 962 A, as there is no teaching or suggestion of the method claimed.

In this regard, Belinga *et al.* teaches the removal of contaminating enzymes, such as adenylate kinase from the firefly luciferase protein using a preparation of firefly luciferase with PEG, followed by affinity chromatography which binds many interfering enzymes of the bioluminescent reaction but not luciferase, which can be obtained without a specific elution step. This problem is quite different from that of the presently claimed invention and therefore it is not believed an ordinarily skilled person would have considered it. In particular, there is no teaching or suggestion of a method according to the presently claimed invention wherein contamination by adenylate kinase is removed by using a mutant adenylate kinase gene which is introduced into a host cell and wherein adenylate kinase is only present in mutant form in the cell and which is denatured on heating or using suitable pH conditions. Furthermore, Belinga *et al* is further distinguished from the presently claimed invention in that it does not teach or suggest the removal of a particular contaminant protein from a culture.

It is also discussed on page 39, first column, third paragraph, that several methods have been reported for the purification of firefly luciferase but that these approaches have certain disadvantages, which are overcome by the described method. There would therefore have been no motivation for an ordinarily skilled person to have attempted to improve the method taught by Belinga *et al.* such that an ordinarily skilled person would not have considered looking elsewhere to improve the same.

Even if an ordinarily skilled person were to have combined EP 0 373 962A with

Belinga *et al.*, the deficiencies of EP 0 373 962A would not have been overcome by Belinga *et al.* as neither teaches or suggests the use of genetic modification to target the removal of specific contaminant proteins. Therefore, the applicants believe that the present invention is inventive and patentable over the combination of these two documents.

Considering US 5 229 285 A [Kayijama *et al.*], this document simply teaches the production of a thermostable luciferase. This problem is quite different from that of the present invention. Therefore the ordinarily skilled person would not have considered this document even if motivated by the other cited art to overcome the deficiencies of the same. However, even if the skilled person were to have considered Kayijama *et al.*, there is no teaching or suggestion of the removal of a contaminant protein when purifying the luciferase and in particular the method of the present invention.

Even if the teaching of Kayijama *et al.* were to have been combined with that of EP 0 373 962A, which is not believed to be likely as there is no motivation in the cited art to have done so, the ordinarily skilled person would still not have produced the method of the present invention as the solution to the deficiencies of EP 0 373 962A are not found in Kayijama *et al.* Accordingly, the presently claimed invention is inventive over Kayijama when taken alone and when combined with EP 0 373 962A and/or Belinga.

Finally, considering Gilles *et al.*, this document teaches that the thermosensitivity of the adenylate kinase from *E. coli* K-12 strain CR341 T28 results from substitution of a serine residue for proline-87 in the wild type enzyme. The enzyme was isolated and

characterised from thermosensitive mutants of *E. coli*. The adenylate kinase was purified from the thermosensitive strain by chromatography and studied to see what changes it caused in the cell. As this paper addresses a very different problem to that of the presently claimed invention, an ordinarily skilled person would not have considered the same.

Even if an ordinarily skilled person were to have considered Gilles *et al.*, there is no teaching or suggestion of the method of the present invention which produces a thermostable luciferase enzyme free of adenylate kinase. In fact no practical application of the teaching is described at all. In addition, the document is an old document (published in 1986) and yet no-one produced the method of the present invention before the applicants' invention. Therefore, the presently claimed invention is clearly inventive over this document when taken alone or in combination with the other cited art.

Even if the teachings of Gilles were to have been combined with EP 0 373 962A, or the other art discussed above, which is not believed to be likely as there was no motivation in the art to have done so, the deficiencies of EP 0 373 962A and/or the other cited art are not cured by Gilles *et al.* In this regard, the inventors have tested the mutant strain of *E. coli* to determine the temperature sensitivity of the adenylate kinase produced by the mutant. It was found, in fact, that the adenylate kinase produced is not particularly temperature sensitive and at 43°C, the activity of the adenylate kinase had not decreased but had in fact increased. (Further details of these tests can be provided upon the Examiner's further request for the same.) This may be because a mutant strain of *E. coli* was used rather than a recombinant one. In any case, an ordinarily

skilled person would therefore have been directed away from the presently claimed invention as by taking such a mutant and using heat in the method as taught by EP 0 373 962A to denature the contaminating adenylate kinase, the contaminating adenylate kinase would not have been removed from the host cell culture. Furthermore, there is no teaching or suggestion of the use of genetic modification to remove a particular protein, in this case adenylate kinase, from a host cell culture containing luciferase. Accordingly, the present invention is inventive over a combination of EP 0 373 962A with Gilles *et al* and the remaining cited art.

The combination of all four documents by the Examiner is considered to be a mosaic of prior art, which cannot be justified or able to establish a *prima facie* case of obviousness as the collection of cited art has been combined with an impermissible use of hindsight knowledge of the presently claimed invention. These documents address different problems and provide different solutions both to each other and to the present invention. There would therefore have been no motivation for an ordinarily skilled person to have combined the documents. An ordinarily skilled person would not therefore have been directed to any of these documents in solving the problem, which the present invention addresses. Even if a skilled person were to have combined all four documents, they would not have produced the presently claimed invention in view of their differences *vis-a-vis* from each other and from the present invention. The applicants submit therefore that the presently claimed invention is inventive and patentable over a combination of the cited documents.

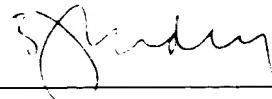
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In view of the above, the claims are submitted to be in condition for allowance and a Notice to that effect is requested.

Respectfully submitted,

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MARKED UP SPECIFICATION

Page 2, delete the paragraph spanning lines 16 through 23 and insert the following therefor:

--One particular application to which luciferase may be put is in an assay for detection of cellular components such as ATP or enzymes such as adenylate kinase (AK), as described in European Patent Application No. 94904295.6. Such assays are useful in detecting the presence of microorganisms in a particular [enviroment] environment. For these purposes, the presence of cellular components which are the target of the assay in the luciferase reagent will produce levels of "background" noise which will have to be taken account of when interpreting results obtained using these products. This is a particular problem in the adenylate kinase assay, which has a high level of sensitivity.--

Page 3, delete the paragraph spanning lines 9 and 10 and insert the following therefor:

--The applicants have devised a new technique where the problem of contamination of products of recombinant DNA technology by [unddesired] undesired or even harmful products can be minimised.--

Page 5, delete the paragraph spanning lines 14 through 19 and insert the following therefor:

--Therefore, an alternative approach is to clone the adenylate kinase gene into a suitable vector such as Promega plasmid "pALTER-1". Site-directed mutagenesis of the amino acids at positions 87 and 107 for example using PCR based methods will

give a gene product which has altered thermolability. Screening of these mutants as described above will indicate which substituent amino acids at these positions give adenylate kinase which is more thermolabile [that] than the native protein.--

Page 5, delete the paragraph spanning line 28 through page 6, line 3 and insert the following therefor:

--Conversely and additionally, the desired polypeptide product may be engineered so that its tolerance to the conditions under which the undesired protein is denatured is increased. For instance, in the case of luciferase enzyme, several thermostable mutants are known in the art and these may be employed in the method of the invention. Alternatively other thermostable mutants or mutants which have increased acid stability etc. can be prepared using similar techniques. [in] In this case, the screening process will select those mutants which have increased tolerance rather than decreased tolerance to the condition being used to denature the undesired polypeptide.--

Page 6, delete the paragraph spanning lines 24-30 and insert the following therefor:

--The invention further provides a method for producing a recombinant cell according to [any one of claims 10 to 14] the present invention which method comprises in any order (a) transforming a host cell with a vector which encodes said undesired protein in a form which is unstable under given conditions, subjecting transformants to said conditions and detecting those in which protein product is denatured, and (b) transforming said host cell with a vector which encodes a desired polypeptide which is

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stable under said conditions and a first selection marker, and using the first selection marker to detect stable transformants.--

Page 7, delete the paragraph spanning lines 15-22 and insert the following therefor:

--The PCR product which will be the wild-type adenylate kinase gene or alternatively a mutant adenylate kinase gene already known to produce thermolabile adenylate kinase such as [stain] strain CV2 is then cloned into a suitable vector, such as the pALTER-1 from Promega, a plasmid based on pBR322. This has disabled antibiotic resistance genes to facilitate mutagenesis. [CV2 is known (Proc. Natl. Acad. Sci. USA, (1970) 65:737) and may be obtained from the *E. coli* Genetic Stock Centre, 355 Osborn Memorial Laboratories, Box 208104, Yale University, New Haven, CT 06520-8104, USA.]--